Gene expression of transcription factor NFATc1 in periodontal diseases

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Abstract: Belibasakis GN, Emingil G, Saygan B, Turkoglu O, Atilla G, Bostanci N. Gene expression of transcription factor NFATc1 in periodontal diseases. APMIS 2011; 119: 167-172. Periodontitis is a disease of infectious aetiology that causes inflammatory destruction of the tooth-supporting tissues. Activated T cells are central to the pathogenesis of the disease, by producing receptor activator of nuclear factor B (NF- B) ligand (RANKL) that stimulates bone resorption. Antigenic activation of T cells is regulated by the induction of transcription factor nuclear factor of activated T cells, cytoplasmic 1 (NFATc1). There is as yet no information on the potential involvement of NFATc1 in periodontal diseases. This study aimed to investigate NFATc1 gene expression levels in periodontal diseases, and analyse the potential correlation with RANKL expression and clinical periodontal parameters. In this cross-sectional study, gingival tissue biopsies were obtained from healthy (n = 10) and periodontally diseased (n = 58) sites. NFATc1 and RANKL gene expression levels in these samples were analysed by quantitative real-time polymerase chain reaction. Compared with healthy subjects, patients with gingivitis, chronic and aggressive periodontitis, exhibited higher NFATc1 expression, which proved to be statistically significant in the periodontitis groups. NFATc1 and RANKL expression levels strongly correlated with each other, and with clinical periodontal parameters. The increased expression of NFATc1 in periodontitis denotes a role for this transcription factor in the pathogenesis of the disease.

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Gene expression of transcription factor NFATc1 in periodontal diseases

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Running head: NFATc1 expression in periodontal disease

SUMMARY

Belibasakis GN, Emingil G, Saygan B, Turkoglu O, Atilla G, Bostanci N. Gene expression of transcription factor NFATc1 in periodontal diseases.

Periodontitis is a disease of infectious aetiology that causes inflammatory destruction of the tooth-supporting tissues. Activated T-cells are central to the pathogenesis of the disease, by producing Receptor activator of NF-?B ligand (RANKL) that stimulates bone resorption. Antigenic activation of T-cells is regulated by the induction of transcription factor Nuclear Factor of Activated T cells, cytoplasmic 1 (NFATc1). There is as yet no information on the potential involvement of NFATc1 in periodontal diseases. This study aimed to investigate NFATc1 gene expression levels in periodontal diseases, and analyse the potential correlation with RANKL expression and clinical periodontal parameters. In this cross-sectional study, gingival tissue biopsies were obtained from healthy (n = 10) and periodontally diseased (n = 58) sites. NFATc1 and RANKL gene expression levels in these samples were analysed by quantitative real-time polymerase chain reaction. Compared to healthy subjects, patients with gingivitis, chronic and aggressive periodontitis, exhibited higher NFATc1 expression, which proved to be statistically significant in the periodontitis groups. NFATc1 and RANKL expression levels strongly correlated with each other, and with clinical periodontal parameters. The increased expression of NFATc1 in periodontitis denotes a role for this transcription factor in the pathogenesis of the disease.

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Key words: NFATc1, RANKL, DC-STAMP, T-cells, periodontal diseases

INTRODUCTION

Periodontitis is characterised by loss of connective tissue attachment and alveolar bone destruction, as a result of inflammation triggered by bacterial biofilms attached on the tooth surface. If the disease is left untreated, it may eventually lead to tooth loss as a result of destruction of the supportive bone. The pathogenesis of the disease largely accounts for a host immune response to the bacterial infection, which is dominated by the presence of T-cells (1, 2). When recruited into the infected area, T-cells can induce bone resorption by producing Receptor Activator of NF-kB Ligand (RANKL), the key osteoclast differentiation cytokine belonging to the tumor necrosis factor ligand family. By activating its cognate RANK receptor on monocytes, RANKL triggers their fusion and differentiation into osteoclasts, an event that can be blocked by its soluble decoy receptor osteoprotegerin (OPG) (3, 4). Activated T-cells appear to be the primary source of RANKL in diseased periodontal tissues (1, 2, 5, 6).

The recognition of bacterial antigens by immuno-receptors on T-cells leads to the production of T-cell-specific cytokines. This event is predominantly mediated by the inducible expression of transcription factor Nuclear Factor of Activated T cells, cytoplasmic 1 (NFATc1, also NFAT2) (7, 8). This transcription factor is expressed at low levels in resting T-cells, but is strongly induced upon activation, in order to enhance and sustain the expression of genes crucial for peripheral T-cell development and function (9). NFATc1 expression is also recently identified in differentiating osteoclasts and is considered to be the major intracellular regulator of osteoclastogenesis downstream of RANKL (10, 11). The dendritic cell–specific transmembrane protein (DC-STAMP) is also induced in osteoclasts upon RANKL stimulation and is involved in their fusion and multi-nucleation (12).

As there is as yet no clinical evidence available on the involvement of NFATc1 and DC-STAMP in periodontal diseases, the present cross-sectional study aimed to investigate their gene expression levels in gingival tissue samples of healthy and periodontally diseased subjects.

MATERIALS AND METHODS

Study population and clinical examination

A total of 58 subjects were included in this study, recruited from the Department of Periodontology, School of Dentistry, Ege University, Izmir (13). The use of human

subjects satisfied the requirements of Ege University Ethics Review Board, and was conducted in accordance to the Declaration of Helsinki. Written and informed consent was obtained from each subject prior to enrolment in the study. Patients were selected according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (14). Complete medical and dental histories were taken. None of the subjects had a history of systemic disease or cigarette smoking, and had not taken medications such as antibiotics or contraceptives that could affect their periodontal status for at least 3 months prior to the study. Clinical periodontal examination included measurement of probing pocket depth (millimeters), clinical attachment level (millimeters) at six sites around each tooth with a manual William's periodontal probe (Hu-Friedy, Chicago, IL), papilla bleeding index (15) and plaque index (16). The healthy group consisted of 3 females and 7 males (16-36, mean age 24.2 ± 8.4 years) with no clinical signs of gingival inflammation or radiographic evidence of alveolar bone loss. The gingivitis group included 4 female and 6 male patients (22-48 years, mean age 34.2 ± 8.7 years), with varying degrees of gingival inflammation and no radiographic evidence of alveolar bone loss. The generalized chronic periodontitis group included 8 females and 10 males (35-61 years, mean age of 46.6 ± 6.2 years). The patients in this group had at least 16 teeth in their mouth, demonstrating generalized (>30% of sites) severe periodontal destruction (clinical attachment loss =5mm), with bleeding on probing scores >50%. The clinical attachment levels were commensurate with the amount of plaque accumulation of the patients. The generalized aggressive periodontitis group consisted of 12 females and 8 males (21-38 years, mean age 28.3 ± 4.6 years). These patients demonstrated a generalized pattern of severe periodontal destruction and clinical attachment loss of = 4 mm on eight or more teeth, at least three of which were other than central incisors or first molars. They also showed severe periodontal tissue destruction and loss of periodontal support inconsistent with their age.

Collection of gingival tissue samples

After screening, the patients were recalled for sampling, prior to entering any therapy phase. A single gingival tissue biopsy was taken from each patient, from interproximal sites of single rooted teeth by gingivectomy, under local anesthesia. These biopsies included both gingival (junctional, crevicular or pocket) epithelium and gingival connective tissue. For the periodontitis groups, samples were collected

from sites with clinical attachment loss =5 mm prior to non-surgical periodontal therapy. Gingival tissue samples from periodontally healthy and gingivitis-affected subjects were obtained during tooth extractions for orthodontic reasons or crown-lengthening procedures. The selected sites had no clinical attachment loss or radiographic evidence of bone loss. One tissue sample from each subject was obtained and was immediately submerged in a sterile tube containing 1 ml RNAlater solution (Ambion Inc, Austin, TX) and stored at +4°C overnight, before long term storage at -40°C until further laboratory analysis.

RNA isolation and Reverse Transcription

Total RNA from gingival tissue biopsies was extracted using the RiboPure RNA Isolation Kit (Ambion Inc, Austin, TX), and quantified using the NanoDrop® ND-1000 spectophotometer. For the reverse transcription reaction, 1 µg of total RNA was incubated with 0.5 µg/ml of oligo dT primer at 70°C for 5 min and then cooled on ice. A master mix was then added to samples, comprising of 200 units moloney murine leukemia virus (M-MLV) reverse transcriptase and buffer, and 10 mM PCR Nucleotide Mix (all from Promega). To obtain the single-stranded cDNA, these samples were then incubated at 40°C for 60 min, 70°C for 15 min, cooled down to +4°C for 5 min, and stored at -20°C until further use.

TaqMan® quantitative real-time PCR (qPCR)

The expression levels of RANKL, NFATc1 and DC-STAMP were investigated by qPCR analysis (ABI Prism 7000 Sequence Detection System, Applied Biosystems). The 18S rRNA was used as an endogenous RNA control in the samples. The standard PCR conditions for template amplification were 10 min at 95°C, followed 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The TaqMan® Gene Expression Assay IDs (Applied Biosystems) were RANKL: Hs00243522-m1, NFATc1: Hs00542675-m1, DC-STAMP: Hs00229255-m1, and 18S rRNA: Hs99999901-s1. The qPCR assay was performed once and each sample was represented in triplicate wells. The relative expression levels of RANKL, NFATc1 and DC-STAMP were calculated by using the comparative Ct method ($2^{-??Ct}$ formula) (17) after normalization to the 18S rRNA. This internal control has been efficiently used in the study of gene expression in gingival tissue biopsies (6).

Statistical analysis

Statistical analyses were performed using non-parametrical methods. Comparisons between all groups were performed using the Kruskal–Wallis test. When there were significant differences ($p<0.05$), comparisons between groups were assessed with Dunn's multiple comparison test. Correlations between RANKL and NFATc1 expression levels, or clinical parameters, were analysed by Spearman's rank correlation. All data analyses were performed using the GraphPad Prism Software 4.0.

RESULTS

Clinical findings of sampling sites

The clinical parameters of the sampled sites are provided in Table 1. The chronic and generalized aggressive periodontitis groups had significantly higher mean probing pocket depth and clinical attachment loss scores than the healthy and gingivitis groups ($p<0.05$). No significant differences were detected between gingivitis and periodontitis groups regarding the papilla bleeding index and plaque index of sampling sites ($p>0.05$).

RANKL and NFATc1 and DC-STAMP gene expression analysis by qPCR

The levels of RANKL, NFATc1 and DC-STAMP gene expression in gingival tissues were evaluated by qPCR analysis. RANKL expression was detected in 4/10 healthy, 8/10 gingivitis, and all chronic periodontitis (18/18) and generalized aggressive periodontitis (20/20) tissue samples (Fig. 1). NFATc1 expression was detected in 8/10 healthy, 9/10 gingivitis, and all chronic periodontitis (18/18) and generalized aggressive periodontitis (20/20) tissue samples (Fig. 2). On the contrary, DC-STAMP expression was sparsely detected (0/0 health, 2/10 gingivitis, 2/18 chronic periodontitis, and 3/20 generalized aggressive periodontitis) among the tissue samples (Fig. 3). Although numerically the relative DC-STAMP expression levels were higher in chronic and generalized aggressive periodontitis ($1.13x10^5$ and $0.66x10^5$, respectively) compared to gingivitis ($0.13x10^5$), the low detection frequency prohibited further statistical analyses.

In comparison to healthy tissues, both RANKL and NFATc1 expressions were higher in all three periodontal diseases groups. However, this difference proved to be significant in the cases of chronic and generalized aggressive periodontitis, but not in

gingivitis. In particular, RANKL expression was higher by 35.6-fold in generalized aggressive periodontitis, 15.8-fold in chronic periodontitis, and 9.3-fold in gingivitis, respectively (Fig. 1). In the case of NFATc1 expression, this was higher by 8.2-fold in generalized aggressive periodontitis, 6.0-fold in chronic periodontitis, and 3.7-fold in gingivitis, respectively (Fig. 2). When the two periodontitis groups were compared, no statistically significant differences were found in RANKL or NFATc1 expression.

The Spearman's rank correlation analysis was then used to investigate the association between RANKL and NFATc1 expression levels in all groups (Fig. 4). A strong positive correlation between the expression of these genes was revealed ($r=0.846$, $p<0.0001$).

Correlation of RANKL and NFATc1 gene expression with clinical parameters

Further analyses were performed in order to test whether there was an association of RANKL and NFATc1 expression levels with site-specific clinical parameters. For this purpose the Spearman's rank correlation analysis was used. The results indicated that the expression levels of both RANKL and NFATc1 were positively correlated ($p<0.01$) with probing pocket depth, clinical attachment levels, papilla bleeding index and plaque index (Table 2).

DISCUSSION

The present study demonstrated that NFATc1 gene expression in gingival tissues is elevated by the severity of periodontal disease, with a significant enhancement in chronic and generalized aggressive periodontitis, compared to health. This could be of potential importance for the pathogenic mechanisms of periodontal disease, as antigenic stimulation of NFATc1 is known to activate the functions of T-cells (8, 9). Hence, increased NFATc1 expression may confirm the increased T-cell activity in periodontitis. However, since NFATc1 is also involved in osteoclastogenesis, the findings could as well denote the presence of differentiating osteoclasts in periodontitis. Despite that the cell source of NFATc1 is not yet clear, this study indicates that NFATc1 up-regulation is an important molecular event in the pathogenesis of periodontitis. The present findings are also in agreement with a recent study demonstrating high NFATc1 expression in experimental periapical bone lesions (18).

RANKL gene expression was also investigated, confirming the general principle that it is elevated in periodontitis, thus strengthening further its role in the pathogenesis of the disease (5, 6, 19, 20). A strong positive correlation was also revealed between the gene expression levels of RANKL and NFATc1, indicating that they are concurrently expressed in periodontal tissues. Activated T-cells are considered the predominant cell type (21) and the major source of RANKL in periodontitis (1, 2, 5, 6). Hence, the findings support the likelihood that NFATc1 gene expression is associated with T-cells, rather than differentiating osteoclasts in the studied gingival tissue samples. This is further corroborated by the very limited detection of DC-STAMP in these samples, a molecule that is associated with the fusion and multi-nucleation of differentiating osteoclasts (12).

Additional analyses were performed to evaluate the correlations of NFATc1 and RANKL gene expression with clinical periodontal parameters. The results indicate that both NFATc1 and RANKL positively correlate with probing pocket depth, clinical attachment loss, papilla bleeding index and plaque index. These results confirm previous findings on RANKL expression (6), and further indicate that NFATc1 as well correlates with clinical parameters of periodontal disease severity.

In conclusion, the present study provides the first evidence for the involvement of NFATc1 in periodontal disease. The increased expression of NFATc1 in periodontitis justifies a further role for this transcription factor in the pathogenesis of the disease. To strengthen further the biological significance of the enhanced NFATc1 gene expression, further studies would be needed, confirming its protein localisation, as well as identifying its cellular source.

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TABLES

Table 1. Clinical parameters of the sampled sites (mean ± standard deviation)

	PPD (mm)	**CAL (mm)**	**PBI**	**PI**
Healthy	1.7 ± 0.63	0	0.3 ± 0.4	0.8 ± 0.9
Gingivitis	2.7 ± 0.6	0	2.6 ± 0.8*	2.2 ± 1.3
CP	6.6 ± 0.8*	7.8 ± 1.7*	3.1 ± 1.7*	2.3 ± 0.7
G-AgP	7.0 ± 1.2*	7.4 ± 1.6*	2.7 ± 1.4*	2.3 ± 0.8

* Significant difference compared to healthy group ($p < 0.05$). PPD, probing pocket depth; CAL, clinical attachment levels; PBI, papilla bleeding index; PI, plaque index; CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis.

Table 2. Correlation values (r) between NFATc1 and RANKL gene expression levels and site-specific clinical parameters.

Clinical Parameters	**NFATc1**	**RANKL**
PPD (mm)	0.374*	0.458*
CAL (mm)	0.422*	0.530*
PI	0.388*	0.413*
PBI	0.479*	0.420*

Spearman's rank correlation analysis was used. A positive correlation was revealed between RANKL or NFATc1 expression levels and all studied clinical parameters (*$p<0.01$). PPD, probing pocket depth; CAL, clinical attachment levels; PI, plaque index; PBI, papilla bleeding index.

FIGURE LEGENDS

Figure 1

Distribution of RANKL gene expression in gingival tissues of healthy subjects (n = 10) and patients with gingivitis (n = 10), chronic periodontitis (n = 18) and generalized aggressive periodontitis (n = 20). The individual values represent the relative gene expression levels of RANKL, measured by qPCR analysis, and normalized against the expression levels of 18S rRNA. The horizontal line represents the mean value in each group. Statistically significantly differences compared to the healthy subject group (*$p < 0.05$).

Figure 2

Distribution of NFATc1 gene expression in gingival tissues of healthy subjects (n = 10) and patients with gingivitis (n = 10), chronic periodontitis (n = 18) and generalized aggressive periodontitis (n = 20). The individual values represent the relative mRNA expression levels of NFATc1, measured by qPCR analysis, and normalized against the expression levels of 18S rRNA. The horizontal line represents the mean value in each group. Statistically significantly differences compared to the healthy subject group (*$p < 0.05$).

Figure 3

Distribution of DC-STAMP gene expression in gingival tissues of healthy subjects (n = 10) and patients with gingivitis (n = 10), chronic periodontitis (n = 18) and generalized aggressive periodontitis (n = 20). The individual values represent the relative mRNA expression levels of DC-STAMP, measured by qPCR analysis, and normalized against the expression levels of 18S rRNA. The horizontal line represents the mean value in each group.

Figure 4

Scatter plot demonstrating a strong positive correlation between RANKL and NFATc1 gene expression in gingival tissues ($r = 0.846$, $p < 0.0001$).

Figure 1

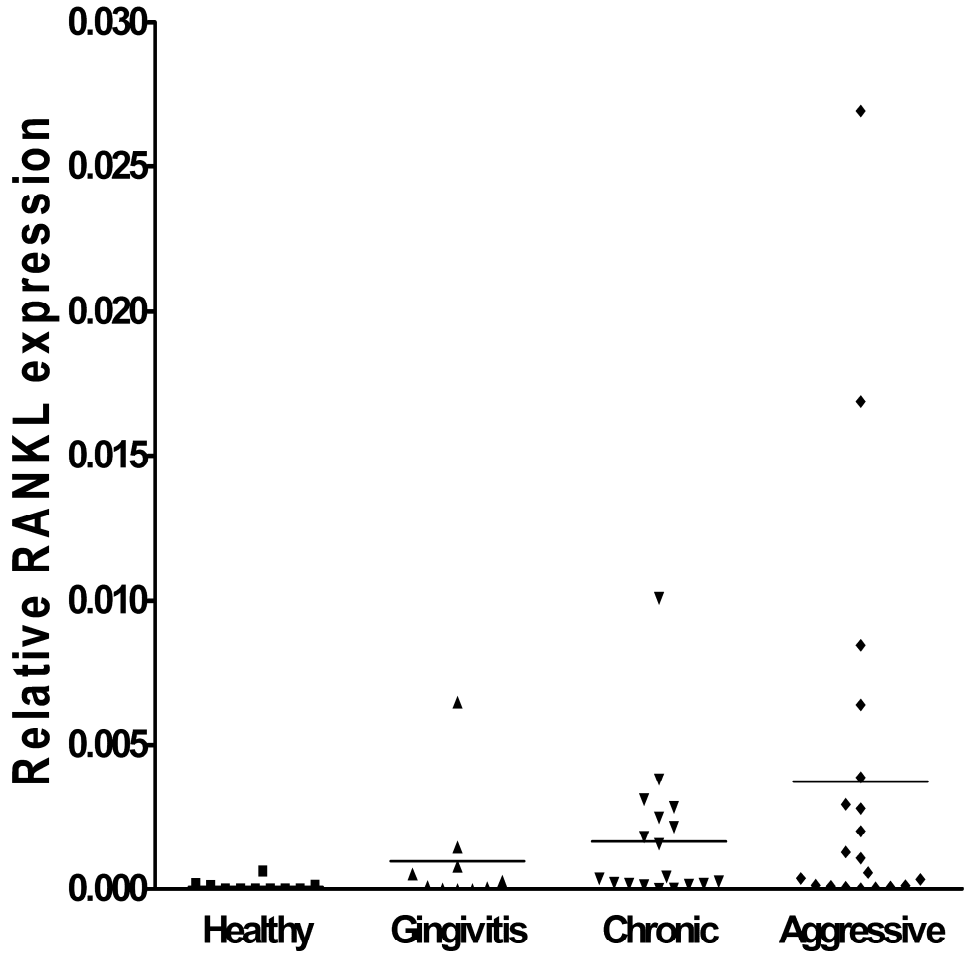

Relative RANKL expression
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Healthy
Gingivitis
Chronic
Aggressive

Figure 2

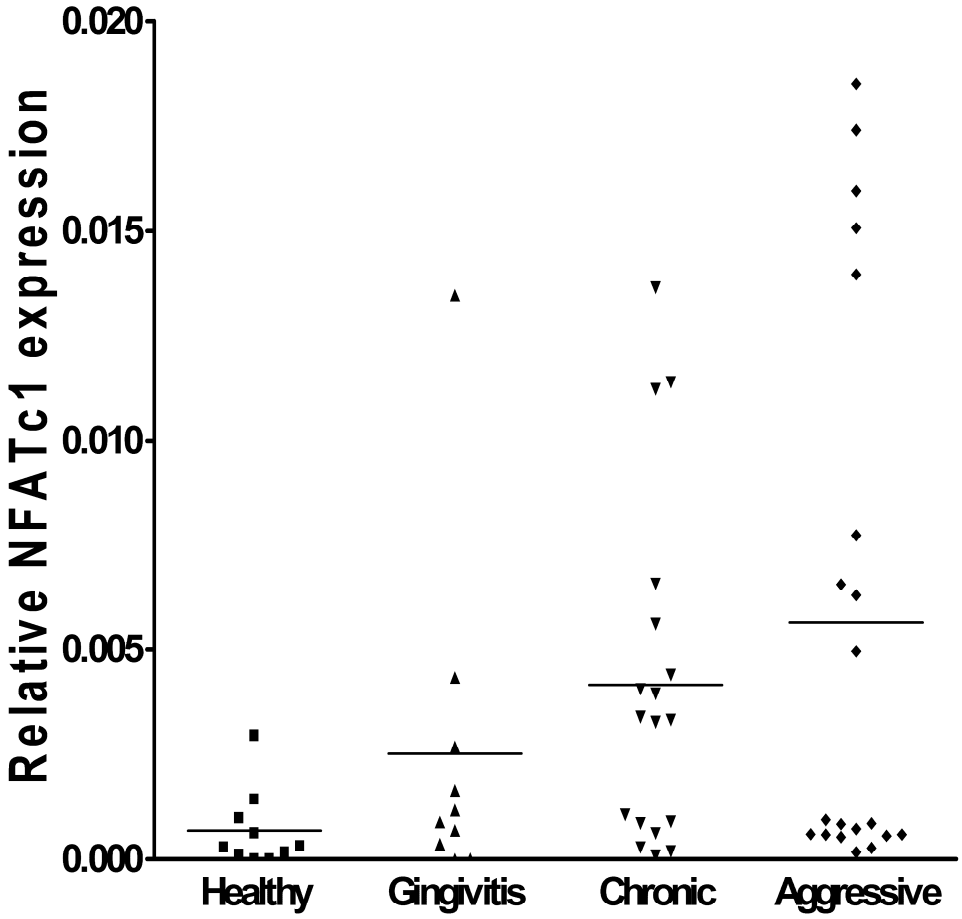

Relative NFATc1 expression
0.020
0.015
0.010
0.005
0.000
Healthy
Gingivitis
Chronic
Aggressive

Figure 3

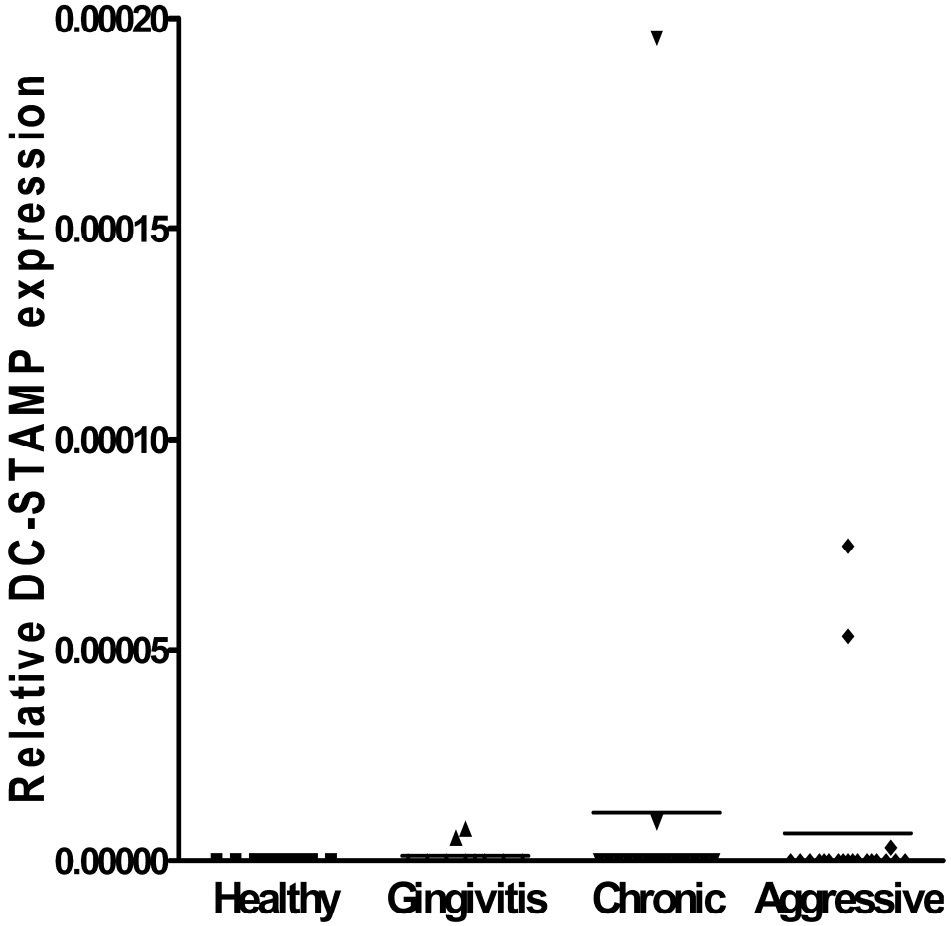
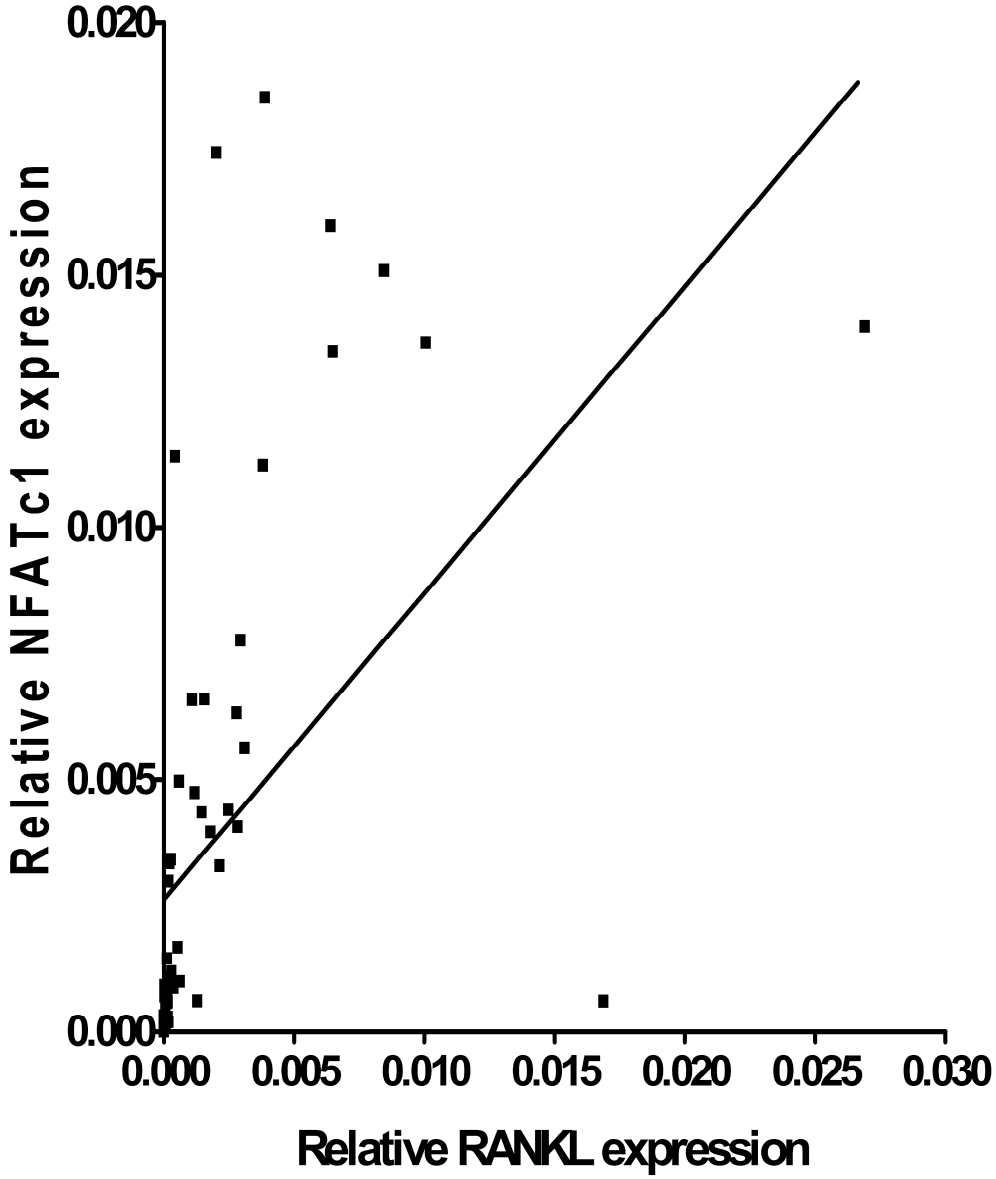

0.00020
0.00015
0.00010
0.00005
0.00000
Relative DC-STAMP expression
Healthy
Gingivitis
Chronic
Aggressive

Figure 4


0.020
0.015
0.010
0.005
0.000
Relative NFATc1 expression
0.000
0.005
0.010
0.015
0.020
0.025
0.030
Relative RANKL expression